

**Fenwick, Robert**

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**From:** Newson, Gloria  
**To:** Fenwick, Robert  
**Cc:** Hawkins, Lynette  
**Subject:** 08/469637  
**Date:** Friday, August 08, 1997 8:10AM

Hi Bob-

I would like to request a copy of 08/469637 (film date 7/21/95) . The examiner is requesting the specs because there is a page missing. I need as soon as possible.

Thank you.

PATENT APPLICATION SERIAL NO. 02/469637

U.S. DEPARTMENT OF COMMERCE  
PATENT AND TRADEMARK OFFICE  
FEE RECORD SHEET

280 TL 06/27/95 08469637  
PTO-1556  
(5/87)

1 101 730.00 CK 325800-381



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

## Application Transmittal Letter

Honorable Commissioner of Patents  
and Trademarks  
Washington, D.C. 20231

Dear Sir:

Transmitted herewith for filing is the patent application:

Inventor(s): Greene et al

For: Human Tumor Necrosis Factor  
Receptor

Enclosed with the application are:

1. Specification (50 pages);
2. Claims (4 pages);
3. Abstract;
4. Five (5) informal sheet(s) of drawing(s);
5. Declaration;
6. Our check number 20549 in the amount of \$730.00.

The fee has been calculated as follows:

	<u>Number of Claims Filed</u>	<u>Extra Claims</u>	<u>Rate</u>	<u>Fee</u>
Basic Fee			\$730	\$ 730.00
Total Claims	20 - 20 =		x 22	\$ .00
Indep. Claims	3 - 3 =		x 76	\$ 0.00
Multiple Dependent Claims Present			\$120	\$ 0.00
<b>TOTAL</b>				<b>\$ 730.00</b>

The Commissioner is authorized to charge payment of any additional filing fees required under 37 CFR 1.16 associated with this communication or credit any overpayment to Deposit Account No. 03-0678.

Respectfully submitted,

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Docket No. 325800-381



#### HUMAN TUMOR NECROSIS FACTOR RECEPTOR

This invention relates to newly identified polynucleotides, polypeptides encoded by such polynucleotides, the use of such polynucleotides and polypeptides, as well as the production of such polynucleotides and polypeptides. The polypeptide of the present invention has been putatively identified as a Tumor Necrosis Factor receptor, and more particularly as a type 2 Tumor Necrosis Factor Receptor. The polypeptide of the present invention will hereinafter be referred to as "TNF receptor". The invention also relates to inhibiting the receptor.

Human tumor necrosis factors  $\alpha$  (TNF- $\alpha$ ) and  $\beta$  (TNF- $\beta$  or lymphotoxin) are related members of a broad class of polypeptide mediators, which includes the interferons, interleukins and growth factors, collectively called cytokines (Beutler, B. and Cerami, A., Annu. Rev. Immunol., 7:625-655 (1989)).

Tumor necrosis factor (TNF- $\alpha$  and TNF- $\beta$ ) was originally discovered as a result of its anti-tumor activity, however, now it is recognized as a pleiotropic cytokine playing important roles in a host of biological processes and pathologies. To date, there are eight known members of the TNF-related cytokine family, TNF- $\alpha$ , TNF- $\beta$  (lymphotoxin- $\alpha$ ),

LT- $\beta$ , and ligands for the Fas receptor, CD30, CD27, CD40 and 4-1BB receptors. These proteins have conserved C-terminal sequences and variable N-terminal sequences which are often used as membrane anchors, with the exception of TNF- $\beta$ . Both TNF- $\alpha$  and TNF- $\beta$  function as homotrimers when they bind to TNF receptors.

TNF is produced by a number of cell types, including monocytes, fibroblasts, T cells, natural killer (NK) cells and predominately by activated macrophages. TNF- $\alpha$  has been reported to have a role in the rapid necrosis of tumors, immunostimulation, autoimmune disease, graft rejection, producing an anti-viral response, septic shock, cerebral malaria, cytotoxicity, protection against deleterious effects of ionizing radiation produced during a course of chemotherapy, such as denaturation of enzymes, lipid peroxidation and DNA damage (Nata et al, J. Immunol. 136(7):2483 (1987)), growth regulation, vascular endothelium effects and metabolic effects. TNF- $\alpha$  also triggers endothelial cells to secrete various factors, including PAI-1, IL-1, GM-CSF and IL-6 to promote cell proliferation. In addition, TNF- $\alpha$  up-regulates various cell adhesion molecules such as E-Selectin, ICAM-1 and VCAM-1. TNF- $\alpha$  and the Fas ligand have also been shown to induce programmed cell death.

A related molecule, lymphotoxin (LT, also referred to as TNF- $\beta$ ), which is produced by activated lymphocytes shows a similar but not identical spectrum of biological activities as TNF. Two different types of LT have been found, LT- $\alpha$  and LT- $\beta$ . LT- $\alpha$  has many activities, including tumor necrosis, induction of an antiviral state, activation of polymorphonuclear leukocytes, induction of class I major histocompatibility complex antigens on endothelial cells, induction of adhesion molecules on endothelium and growth hormone stimulation (Ruddle, N. and Homer, R., Prog. Allergy, 40:162-182 (1988)).

The first step in the induction of the various cellular responses mediated by TNF or LT is their binding to specific cell surface or soluble receptors. Two distinct TNF receptors of approximately 55-KDa (TNF-R1) and 75-KDa (TNF-R2) have been identified (Hohman, H.P. et al., J. Biol. Chem., 264:14927-14934 (1989)), and human and mouse cDNAs corresponding to both receptor types have been isolated and characterized (Loetscher, H. et al., Cell, 61:351 (1990)). Both TNF-Rs share the typical structure of cell surface receptors including extracellular, transmembrane and intracellular regions.

These molecules exist not only in cell bound forms, but also in soluble forms, consisting of the cleaved extracellular domains of the intact receptors (Nophar et al., EMBO Journal, 9 (10):3269-76 (1990)). The extracellular domains of TNF-R1 and TNF-R2 share 28% identity and are characterized by four repeated cysteine-rich motifs with significant intersubunit sequence homology. The majority of cell types and tissues appear to express both TNF receptors and both receptors are active in signal transduction, however, they are able to mediate distinct cellular responses. Further, TNF-R2 was shown to exclusively mediate human T cell proliferation by TNF as shown in PCT WO 94/09137.

TNF-R1 dependent responses include accumulation of C-FOS, IL-6, and manganese superoxide dismutase mRNA, prostaglandin E2 synthesis, IL-2 receptor and MHC class I and II cell surface antigen expression, growth inhibition, and cytotoxicity. TNF-R1 also triggers second messenger systems such as phospholipase A<sub>2</sub>, protein kinase C, phosphatidylcholine-specific phospholipase C and sphingomyelinase (Pfefferk et al., Cell, 73:457-467 (1993)).

The receptor polypeptide of the present invention binds TNF, and in particular, TNF- $\beta$ . Further, the TNF receptor may also bind other ligands, including but not limited to Nerve Growth Factor, due to homology to a family of receptors and

antigens which are involved in other critical biological processes. This family shows highly conserved cysteine residues and includes the low affinity NGF receptor, which plays an important role in the regulation of growth and differentiation of nerve cells, the Fas receptor also called APO, a receptor which is involved in signalling for apoptosis and which, based on a study with mice deficient in its function, seems to play an important role in the etiology of a lupus-like disease, the TNF-R1, the B cell antigen CD40, and the T cell activation antigen CD27.

In accordance with one aspect of the present invention, there is provided a novel mature polypeptide which is a putative TNF receptor, as well as fragments, analogs and derivatives thereof. The polypeptide of the present invention is of human origin.

In accordance with another aspect of the present invention, there are provided isolated nucleic acid molecules encoding the polypeptide of the present invention, including mRNAs, DNAs, cDNAs, genomic DNA as well as antisense analogs thereof and biologically active and diagnostically or therapeutically useful fragments thereof.

In accordance with yet a further aspect of the present invention, there is provided a process for producing such polypeptides by recombinant techniques which comprises culturing recombinant prokaryotic and/or eukaryotic host cells, containing a nucleic acid sequence encoding a polypeptide of the present invention, under conditions promoting expression of said protein and subsequent recovery of said protein.

In accordance with yet a further aspect of the present invention, there is provided a process for utilizing such polypeptide, or polynucleotide encoding such polypeptide to screen for receptor antagonists and/or agonists and/or receptor ligands.



In accordance with yet a further aspect of the present invention, there are provided nucleic acid probes comprising nucleic acid molecules of sufficient length to specifically hybridize to the polypeptide of the present invention.

In accordance with still another aspect of the present invention, there is provided a process of using such agonists for treating conditions related to insufficient TNF receptor activity, for example, to inhibit tumor growth, to stimulate human cellular proliferation, e.g., T-cell proliferation, to regulate the immune response and antiviral responses, to protect against the effects of ionizing radiation, to protect against chlamidia infection, to regulate growth and to treat immunodeficiencies such as is found in HIV.

In accordance with another aspect of the present invention, there is provided a process of using such antagonists for treating conditions associated with over-expression of the TNF receptor, for example, for treating T-cell mediated autoimmune diseases such as AIDS, septic shock, cerebral malaria, graft rejection, cytotoxicity, cachexia, apoptosis and inflammation.

These and other aspects of the present invention should be apparent to those skilled in the art from the teachings herein.

The following drawings are illustrative of embodiments of the invention and are not meant to limit the scope of the invention as encompassed by the claims.

Figure 1 shows the cDNA sequence and corresponding deduced amino acid sequence of the polypeptide of the present invention. The initial 21 amino acids represent the putative leader sequence and are underlined. The standard one-letter abbreviations for amino acids are used. Sequencing was performed using a 373 automated DNA sequencer (Applied Biosystems, Inc.). Sequencing accuracy is predicted to be greater than 97% accurate.

Figure 2 illustrates an amino acid sequence alignment of the polypeptide of the present invention (upper line) and the human type 2 TNF receptor (lower line).

The term "gene" or "cistron" means the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons).

In accordance with an aspect of the present invention, there is provided an isolated nucleic acid (polynucleotide) which encodes for the mature polypeptide having the deduced amino acid sequence of Figure 1 (SEQ ID No. 2) or for the mature polypeptide encoded by the cDNA of the clone deposited as ATCC Deposit No. 75899 on September 28, 1994.

A polynucleotide encoding a polypeptide of the present invention may be obtained from human pulmonary tissue, hippocampus and adult heart. The polynucleotide of this invention was discovered in a cDNA library derived from human early passage fibroblasts (HSA 172 cells). It is structurally related to the human TNF-R2 receptor. It contains an open reading frame encoding a protein of 390 amino acid residues of which approximately the first 21 amino acids residues are the putative leader sequence such that the mature protein comprises 369 amino acids. The protein exhibits the highest degree of homology to a human type 2 TNF receptor with 39% identity and 46% similarity over an 88 amino acid stretch. Six conserved cyteines present in modules of 40 residues in all TNF receptors are conserved in this receptor.

The TNF receptor of the present invention is a soluble receptor and is secreted, however, it may also exist as a membrane bound receptor having a transmembrane region and an intra- and extracellular region. The polypeptide of the present invention may bind TNF and lymphotoxin ligands.

In accordance with an aspect of the present invention there is provided a polynucleotide which may be in the form of RNA or in the form of DNA, which DNA includes cDNA, genomic DNA, and synthetic DNA. The DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand. The coding sequence which encodes the mature polypeptide may be identical to the coding sequence shown in Figure 1 (SEQ ID No. 1) or that of the deposited clone or may be a different coding sequence which coding sequence, as a result of the redundancy or degeneracy of the genetic code, encodes the same mature polypeptide as the DNA of Figure 1 (SEQ ID No. 1) or the deposited cDNA.

The polynucleotide which encodes for the mature polypeptide of Figure 1 (SEQ ID No. 2) or for the mature polypeptide encoded by the deposited cDNA may include: only the coding sequence for the mature polypeptide; the coding sequence for the mature polypeptide and additional coding sequence such as a leader or secretory sequence or a proprotein sequence; the coding sequence for the mature polypeptide (and optionally additional coding sequence) and non-coding sequence, such as introns or non-coding sequence 5' and/or 3' of the coding sequence for the mature polypeptide.

Thus, the term "polynucleotide encoding a polypeptide" encompasses a polynucleotide which includes only coding sequence for the polypeptide as well as a polynucleotide which includes additional coding and/or non-coding sequence.

The present invention further relates to variants of the hereinabove described polynucleotides which encode for fragments, analogs and derivatives of the polypeptide having the deduced amino acid sequence of Figure 1 (SEQ ID No. 2) or the polypeptide encoded by the cDNA of the deposited clone. The variant of the polynucleotide may be a naturally

occurring allelic variant of the polynucleotide or a non-naturally occurring variant of the polynucleotide.

Thus, the present invention includes polynucleotides encoding the same mature polypeptide as shown in Figure 1 (SEQ ID No. 2) or the same mature polypeptide encoded by the cDNA of the deposited clone as well as variants of such polynucleotides which variants encode for a fragment, derivative or analog of the polypeptide of Figure 1 (SEQ ID No. 2) or the polypeptide encoded by the cDNA of the deposited clone. Such nucleotide variants include deletion variants, substitution variants and addition or insertion variants.

As hereinabove indicated, the polynucleotide may have a coding sequence which is a naturally occurring allelic variant of the coding sequence shown in Figure 1 (SEQ ID No. 1) or of the coding sequence of the deposited clone. As known in the art, an allelic variant is an alternate form of a polynucleotide sequence which may have a substitution, deletion or addition of one or more nucleotides, which does not substantially alter the function of the encoded polypeptide.

The present invention also includes polynucleotides, wherein the coding sequence for the mature polypeptide may be fused in the same reading frame to a polynucleotide sequence which aids in expression and secretion of a polypeptide from a host cell, for example, a leader sequence which functions as a secretory sequence for controlling transport of a polypeptide from the cell. The polypeptide having a leader sequence is a preprotein and may have the leader sequence cleaved by the host cell to form the mature form of the polypeptide. The polynucleotides may also encode for a proprotein which is the mature protein plus additional 5' amino acid residues. A mature protein having a prosequence is a proprotein and is an inactive form of the protein. Once the prosequence is cleaved an active mature protein remains.

Thus, for example, the polynucleotide of the present invention may encode for a mature protein, or for a protein having a prosequence or for a protein having both a prosequence and a presequence (leader sequence).

The polynucleotides of the present invention may also have the coding sequence fused in frame to a marker sequence which allows for purification of the polypeptide of the present invention. The marker sequence may be a hexahistidine tag supplied by a pQE-9 vector to provide for purification of the mature polypeptide fused to the marker in the case of a bacterial host, or, for example, the marker sequence may be a hemagglutinin (HA) tag when a mammalian host, e.g. COS-7 cells, is used. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson, I., et al., Cell, 37:767 (1984)). The coding sequence may also be fused to a sequence which codes for a fusion protein such as an IgG Fc fusion protein.

The term "gene" means the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons).

Fragments of the full length gene of the present invention may be used as a hybridization probe for a cDNA library to isolate the full length cDNA and to isolate other cDNAs which have a high sequence similarity to the gene or similar biological activity. Probes of this type preferably have at least 30 bases and may contain, for example, 50 or more bases. The probe may also be used to identify a cDNA clone corresponding to a full length transcript and a genomic clone or clones that contain the complete gene including regulatory and promotor regions, exons, and introns. An example of a screen comprises isolating the coding region of the gene by using the known DNA sequence to synthesize an oligonucleotide probe. Labeled oligonucleotides having a

sequence complementary to that of the gene of the present invention are used to screen a library of human cDNA, genomic DNA or mRNA to determine which members of the library the probe hybridizes to.

The present invention further relates to polynucleotides which hybridize to the hereinabove-described sequences if there is at least 70%, preferably at least 90%, and more preferably at least 95% identity between the sequences. The present invention particularly relates to polynucleotides which hybridize under stringent conditions to the hereinabove-described polynucleotides. As herein used, the term "stringent conditions" means hybridization will occur only if there is at least 95% and preferably at least 97% identity between the sequences. The polynucleotides which hybridize to the hereinabove described polynucleotides in a preferred embodiment encode polypeptides which either retain substantially the same biological function or activity as the mature polypeptide encoded by the cDNAs of Figure 1 (SEQ ID NO:1) or the deposited cDNA(s).

Alternatively, the polynucleotide may have at least 20 bases, preferably 30 bases, and more preferably at least 50 bases which hybridize to a polynucleotide of the present invention and which has an identity thereto, as hereinabove described, and which may or may not retain activity. For example, such polynucleotides may be employed as probes for the polynucleotide of SEQ ID NO:1, for example, for recovery of the polynucleotide or as a diagnostic probe or as a PCR primer.

Thus, the present invention is directed to polynucleotides having at least a 70% identity, preferably at least 90% and more preferably at least a 95% identity to a polynucleotide which encodes the polypeptide of SEQ ID NO:2 as well as fragments thereof, which fragments have at least 30 bases and preferably at least 50 bases and to polypeptides encoded by such polynucleotides. The deposit(s) referred to

one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to the mature polypeptide, such as an IgG Fc fusion region peptide or leader or secretory sequence or a sequence which is employed for purification of the mature polypeptide or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

The polypeptides and polynucleotides of the present invention are preferably provided in an isolated form, and preferably are purified to homogeneity.

The term "isolated" means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or polypeptide, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

The polypeptides of the present invention include the polypeptide of SEQ ID NO:2 (in particular the mature polypeptide) as well as polypeptides which have at least 70% similarity (preferably at least 70% identity) to the polypeptide of SEQ ID NO:2 and more preferably at least 90% similarity (more preferably at least 90% identity) to the polypeptide of SEQ ID NO:2 and still more preferably at least 95% similarity (still more preferably at least 90% identity) to the polypeptide of SEQ ID NO:2 and also include portions of such polypeptides with such portion of the polypeptide.

generally containing at least 30 amino acids and more preferably at least 50 amino acids.

As known in the art "similarity" between two polypeptides is determined by comparing the amino acid sequence and its conserved amino acid substitutes of one polypeptide to the sequence of a second polypeptide.

Fragments or portions of the polypeptides of the present invention may be employed for producing the corresponding full-length polypeptide by peptide synthesis; therefore, the fragments may be employed as intermediates for producing the full-length polypeptides. Fragments or portions of the polynucleotides of the present invention may be used to synthesize full-length polynucleotides of the present invention.

The present invention also relates to vectors which include polynucleotides of the present invention, host cells which are genetically engineered with vectors of the invention and the production of polypeptides of the invention by recombinant techniques.

Host cells are genetically engineered (transduced or transformed or transfected) with the vectors of this invention which may be, for example, a cloning vector or an expression vector. The vector may be, for example, in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the nucleic acid sequences of the present invention. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The polynucleotides of the present invention may be employed for producing polypeptides by recombinant techniques. Thus, for example, the polynucleotide may be included in any one of a variety of expression vectors for



expressing a polypeptide. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences, e.g., derivatives of SV40; bacterial plasmids; phage DNA; baculovirus; yeast plasmids; vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. However, any other vector may be used as long as it is replicable and viable in the host.

The appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by procedures known in the art. Such procedures and others are deemed to be within the scope of those skilled in the art.

The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct mRNA synthesis. As representative examples of such promoters, there may be mentioned: LTR or SV40 promoter, the E. coli lac or trp, the phage lambda P<sub>L</sub> promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression.

In addition, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in E. coli.

The vector containing the appropriate DNA sequence as hereinabove described, as well as an appropriate promoter or control sequence, may be employed to transform an appropriate host to permit the host to express the protein.

As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as E. coli, Streptomyces, Salmonella typhimurium; fungal cells, such as yeast; insect cells such as Drosophila S2 and Spodoptera Sf9; animal cells such as CHO, COS or Bowes melanoma; adenoviruses; plant cells, etc. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

More particularly, the present invention also includes recombinant constructs comprising one or more of the sequences as broadly described above. The constructs comprise a vector, such as a plasmid or viral vector, into which a sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment, the construct further comprises regulatory sequences, including, for example, a promoter, operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example. Bacterial: pQE70, pQE60, pQE-9 (Qiagen), pBS, pD10, phagescript, psiX174, pbluescript SK, pbsks, pNH8A, pNH16a, pNH18A, pNH46A (Stratagene); pTRC99a, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia). Eukaryotic: pWLNEO, pSV2CAT, pOG44, pXT1, pSG (Stratagene) pSVK3, pBPV, pMSG, pSVL (Pharmacia). However, any other plasmid or vector may be used as long as they are replicable and viable in the host.

Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers. Two appropriate vectors are pKK232-8 and pCM7. Particular named bacterial promoters include lacI, lacZ, T3, T7, gpt, lambda P<sub>R</sub>, P<sub>L</sub> and trp. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate

vector and promoter is well within the level of ordinary skill in the art.

In a further embodiment, the present invention relates to host cells containing the above-described constructs. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation (Davis, I., Dibner, M., Battey, I., Basic Methods in Molecular Biology, (1986)).

The constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Alternatively, the polypeptides of the invention can be synthetically produced by conventional peptide synthesizers.

Mature proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook, et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, N.Y., (1989), the disclosure of which is hereby incorporated by reference.

Transcription of the DNA encoding the polypeptides of the present invention by higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp that act on a promoter to increase its transcription. Examples including the SV40 enhancer on the late side of the replication origin bp 100 to 270, a cytomegalovirus early

promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, e.g., the ampicillin resistance gene of E. coli and S. cerevisiae TRP1 gene, and a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK),  $\alpha$ -factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein into the periplasmic space or extracellular medium. Optionally, the heterologous sequence can encode a fusion protein including an N-terminal identification peptide imparting desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product.

Useful expression vectors for bacterial use are constructed by inserting a structural DNA sequence encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector and to, if desirable, provide amplification within the host. Suitable prokaryotic hosts for transformation include E. coli, Bacillus subtilis, Salmonella typhimurium and various species within the genera Pseudomonas, Streptomyces, and Staphylococcus, although others may also be employed as a matter of choice.

As a representative but nonlimiting example, useful expression vectors for bacterial use can comprise a

selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GEM1 (Promega Biotec, Madison, WI, USA). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed.

Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter is induced by appropriate means (e.g., temperature shift or chemical induction) and cells are cultured for an additional period.

Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification.

Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents, such methods are well known to those skilled in the art.

Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman, Cell, 23:175 (1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

The polypeptide of the present invention can be recovered and purified from recombinant cell cultures by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Protein refolding steps can be used, as necessary, in completing configuration of the mature protein. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

The polypeptides of the present invention may be a naturally purified product, or a product of chemical synthetic procedures, or produced by recombinant techniques from a prokaryotic or eukaryotic host (for example, by bacterial, yeast, higher plant, insect and mammalian cells in culture). Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. Polypeptides of the invention may also include an initial methionine amino acid residue.

The TNF receptor of the present invention was assayed for the ability to bind TNF- $\alpha$  and TNF- $\beta$ , however, the present invention also contemplates the ability of the receptor to bind other TNF-like proteins. Monoclonal antibodies specific to TNF- $\alpha$  and TNF- $\beta$  were prepared. These monoclonal antibodies were bound to TNF- $\alpha$  and TNF- $\beta$  and a control ELISA assay was performed to quantify the amount of monoclonal antibody present. The TNF receptor was then bound to TNF- $\alpha$  and TNF- $\beta$  in the same way in which the monoclonal antibody was bound and another ELISA assay was performed. The TNF receptor was found to bind to TNF- $\beta$  just as strongly as the monoclonal antibody, while it only bound TNF- $\alpha$  two-thirds as strongly.

Fragments of the full length polynucleotide sequences of the present invention may be used as a hybridization probe for a cDNA library to isolate other genes which have a high sequence similarity to the polynucleotide sequence of the present invention or similar biological activity. Probes of this type generally have at least 50 bases, although they may have a greater number of bases. The probe may also be used as markers to identify a cDNA clone corresponding to a full length transcript and a genomic clone or clones that contain the complete polynucleotide sequence of the present invention including regulatory and promotor regions, exons, and introns. An example of a screen comprises isolating the coding region of the gene of the present invention by using the known DNA sequence to synthesize an oligonucleotide probe. Labeled oligonucleotides having a sequence complementary to that of the gene of the present invention are used to screen a library of human cDNA, genomic DNA or mRNA to determine which members of the library the probe hybridizes to.

This invention also provides a method of screening compounds to identify compounds which interact with the polypeptide of the present invention which comprises contacting a mammalian cell comprising an isolated DNA molecule encoding and expressing a the polypeptide of the present invention with a plurality of compounds, determining those which activate or block the activation of the receptor, and thereby identifying compounds which specifically interact with, and activate or block the activation of the polypeptide of the present invention.

This invention also contemplates the use of the polynucleotide of the present invention as a diagnostic. For example, if a mutation is present, conditions would result from a lack of TNF receptor activity. Further, mutations which enhance TNF receptor activity would lead to diseases associated with an over-expression of the receptor, e.g.,

endotoxic shock. Mutated genes can be detected by comparing the sequence of the defective gene with that of a normal one. Subsequently one can verify that a mutant gene is associated with a disease condition or the susceptibility to a disease condition. That is, a mutant gene which leads to the underexpression of the TNF receptor would be associated with an inability of TNF to inhibit tumor growth.

Individuals carrying mutations in the polynucleotide of the present invention may be detected at the DNA level by a variety of techniques. Nucleic acids used for diagnosis may be obtained from a patient's cells which include, but are not limited to, blood, urine, saliva and tissue biopsy. The genomic DNA may be used directly for detection or may be amplified enzymatically by using PCR (Saiki et al., Nature, 324:163-166 (1986)) prior to analysis. RNA or cDNA may also be used for the same purpose. As an example, PCR primers complementary to the nucleic acid of the instant invention can be used to identify and analyze gene mutations. For example, deletions and insertions can be detected by a change in the size of the amplified product in comparison to the normal genotype. Point mutations can be identified by hybridizing amplified DNA to radiolabeled RNA or alternatively, radiolabeled TNF receptor antisense DNA sequences. Perfectly matched sequences can be distinguished from mismatched duplexes by RNase A digestion or by differences in melting temperatures. Such a diagnostic would be particularly useful for prenatal or even neonatal testing.

Sequence differences between the reference gene and "mutants" may be revealed by the direct DNA sequencing method. In addition, cloned DNA segments may be used as probes to detect specific DNA segments. The sensitivity of this method is greatly enhanced when combined with PCR. For example, a sequencing primary used with double stranded PCR product or a single stranded template molecule generated by a modified PCR product. The sequence determination is



performed by conventional procedures with radiolabeled nucleotides or by automatic sequencing procedures with fluorescent tags.

Sequence changes at the specific locations may be revealed by nuclease protection assays, such as RNase and S1 protection or the chemical cleavage method (for example, Cotton et al., PNAS, 85:4397-4401 (1985)).

The present invention further relates to a diagnostic assay which detects an altered level of a soluble form of the polypeptide of the present invention where an elevated level in a sample derived from a host is indicative of certain diseases. Assays available to detect levels of soluble receptors are well known to those of skill in the art, for example, radioimmunoassays, competitive-binding assays, Western blot analysis, and preferably an ELISA assay may be employed.

An ELISA assay initially comprises preparing an antibody specific to an antigen to the polypeptide of the present invention, preferably a monoclonal antibody. In addition a reporter antibody is prepared against the monoclonal antibody. To the reporter antibody is attached a detectable reagent such as radioactivity, fluorescence or in this example a horseradish peroxidase enzyme. A sample is now removed from a host and incubated on a solid support, e.g. a polystyrene dish, that binds the proteins in the sample. Any free protein binding sites on the dish are then covered by incubating with a non-specific protein such as bovine serum albumen. Next, the monoclonal antibody is incubated in the dish during which time the monoclonal antibodies attach to any proteins of the present invention which are attached to the polystyrene dish. All unbound monoclonal antibody is washed out with buffer. The reporter antibody linked to horseradish peroxidase is now placed in the dish resulting in binding of the reporter antibody to any monoclonal antibody bound to the polypeptide of the present invention.

Unattached reporter antibody is then washed out. Peroxidase substrates are then added to the dish and the amount of color developed in a given time period is a measurement of the amount of the protein of interest present in a given volume of patient sample when compared against a standard curve.

A competition assay may be employed wherein antibodies specific to the polypeptides of the present invention are attached to a solid support. Labeled TNF receptor polypeptides, and a sample derived from the host are passed over the solid support and the amount of label detected attached to the solid support can be correlated to a quantity in the sample. The soluble form of the receptor may also be employed to identify agonists and antagonists.

A thymocyte proliferation assay may be employed to identify both ligands and potential agonists and antagonists to the polypeptide of the present invention. For example, thymus cells are disaggregated from tissue and grown in culture medium. Incorporation of DNA precursors such as <sup>3</sup>H-thymidine or 5-bromo-2'-deoxyuridine (BrdU) is monitored as a parameter for DNA synthesis and cellular proliferation. Cells which have incorporated BrdU into DNA can be detected using a monoclonal antibody against BrdU and measured by an enzyme or fluorochrome-conjugated second antibody. The reaction is quantitated by fluorimetry or by spectrophotometry. Two control wells and an experimental well are set up. TNF- $\beta$  is added to all wells, while soluble receptors of the present invention are added to the experimental well. Also added to the experimental well is a compound to be screened. The ability of the compound to be screened to inhibit the interaction of TNF- $\beta$  with the receptor polypeptides of the present invention may then be quantified. In the case of the agonists, the ability of the compound to enhance this interaction is quantified.

A determination may be made whether a ligand not known to be capable of binding to the polypeptide of the present

invention can bind thereto comprising contacting a mammalian cell comprising an isolated molecule encoding a polypeptide of the present invention with a ligand under conditions permitting binding of ligands known to bind thereto, detecting the presence of any bound ligand, and thereby determining whether such ligands bind to a polypeptide of the present invention. Also, a soluble form of the receptor may be utilized in the above assay where it is secreted in to the extra-cellular medium and contacted with ligands to determine which will bind to the soluble form of the receptor.

Other agonist and antagonist screening procedures involve providing appropriate cells which express the receptor on the surface thereof. In particular, a polynucleotide encoding a polypeptide of the present invention is employed to transfect cells to thereby express the polypeptide. Such transfection may be accomplished by procedures as hereinabove described.

Thus, for example, such assay may be employed for screening for a receptor antagonist by contacting the cells which encode the polypeptide of the present invention with both the receptor ligand and a compound to be screened. Inhibition of the signal generated by the ligand indicates that a compound is a potential antagonist for the receptor, i.e., inhibits activation of the receptor.

The screening may be employed for determining an agonist by contacting such cells with compounds to be screened and determining whether such compounds generate a signal, i.e., activates the receptor.

Other screening techniques include the use of cells which express the polypeptide of the present invention (for example, transfected CHO cells) in a system which measures extracellular pH changes caused by receptor activation, for example, as described in Science, Volume 246, pages 181-296 (1989). In another example, potential agonists or antagonists may be contacted with a cell which expresses the

polypeptide of the present invention and a second messenger response, e.g., signal transduction may be measured to determine whether the potential antagonist or agonist is effective.

Another screening technique involves expressing the receptor polypeptide wherein it is linked to phospholipase C or D. As representative examples of such cells, there may be mentioned endothelial cells, smooth muscle cells, embryonic kidney cells and the like. The screening for an antagonist or agonist may be accomplished as hereinabove described by detecting activation of the receptor or inhibition of activation of the receptor from the phospholipase second signal.

Antibodies may be utilized as both an agonist and antagonist depending on which part of the polypeptide of the present invention the antibody binds to. The antibody in one instance can bind to the active site and block ligand access. However, it has been observed that monoclonal antibodies directed against certain TNF receptors can act as specific agonists when binding to the extra-cellular domain of the receptor.

In addition to the antagonists identified above, oligonucleotides which bind to the TNF receptor may also act as TNF receptor antagonists. Alternatively, a potential TNF receptor antagonist may be a soluble form of the TNF receptor which contains the complete extra-cellular region of the TNF receptor and which binds to ligands to inhibit their biological activity.

Another potential TNF receptor antagonist is an antisense construct prepared using antisense technology. Antisense technology can be used to control gene expression through triple-helix formation or antisense DNA or RNA, both of which methods are based on binding of a polynucleotide to DNA or RNA. For example, the 5' coding portion of the polynucleotide sequence, which encodes for the mature

polypeptides of the present invention, is used to design an antisense RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription (triple helix -see Lee et al., Nucl. Acids Res., 6:3073 (1979); Cooney et al, Science, 241:456 (1988); and Dervan et al., Science, 251: 1360 (1991)), thereby preventing transcription and the production of TNF receptors. The antisense RNA oligonucleotide hybridizes to the mRNA in vivo and blocks translation of the mRNA molecule into the TNF receptor polypeptide (antisense - Okano, J. Neurochem., 56:560 (1991); Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988)). The oligonucleotides described above can also be delivered to cells such that the antisense RNA or DNA may be expressed in vivo to inhibit production of TNF receptors.

TNF receptor antagonists also include a small molecule which binds to and occupies the TNF receptor thereby making the receptor inaccessible to ligands which bind thereto such that normal biological activity is prevented. Examples of small molecules include but are not limited to small peptides or peptide-like molecules.

The TNF receptor agonists may be employed to stimulate ligand activities, such as inhibition of tumor growth and necrosis of certain transplantable tumors. The agonists may also be employed to stimulate cellular differentiation, for example, T-cell, fibroblasts and haemopoietic cell differentiation. Agonists to the TNF receptor may also augment TNF's role in the host's defense against microorganisms and prevent related diseases (infections such as that from *L. monocytogenes*) and chlamidiae. The agonists may also be employed to protect against the deleterious effects of ionizing radiation produced during a course of radiotherapy, such as denaturation of enzymes, lipid peroxidation, and DNA damage.

The agonists may also be employed to mediate an anti-viral response, to regulate growth, to mediate the immune response and to treat immunodeficiencies related to diseases such as HIV.

Antagonists to the TNF receptor may be employed to treat autoimmune diseases, for example, graft versus host rejection and allograft rejection, and T-cell mediated autoimmune diseases such as AIDS. It has been shown that T-cell proliferation is stimulated via a type 2 TNF receptor. Accordingly, antagonizing the receptor may prevent the proliferation of T-cells and treat T-cell mediated autoimmune diseases.

The antagonists may also be employed to prevent apoptosis, which is the basis for diseases such as viral infection, rheumatoid arthritis, systemic lupus erythematosus, insulin-dependent diabetes mellitus, and graft rejection. Similarly, the antagonists may be employed to prevent cytotoxicity.

The antagonists to the TNF receptor may also be employed to treat B cell cancers which are stimulated by TNF.

Antagonists to the TNF receptor may also be employed to treat and/or prevent septic shock, which remains a critical clinical condition. Septic shock results from an exaggerated host response, mediated by protein factors such as TNF and IL-1, rather than from a pathogen directly. For example, lipopolysaccharides have been shown to elicit the release of TNF leading to a strong and transient increase of its serum concentration. TNF causes shock and tissue injury when administered in excessive amounts. Accordingly, antagonists to the TNF receptor will block the actions of TNF and treat/prevent septic shock. These antagonists may also be employed to treat meningococcemia in children which correlates with high serum levels of TNF.

Among other disorders which may be treated by the antagonists to TNF receptors, there are included,

inflammation which is mediated by TNF receptor ligands, and the bacterial infections cachexia and cerebral malaria. The TNF receptor antagonists may also be employed to treat inflammation mediated by ligands to the receptor such as TNF.

The soluble TNF receptor and agonists and antagonists may be employed in combination with a suitable pharmaceutical carrier. Such compositions comprise a therapeutically effective amount of the soluble receptor or agonist or antagonist, and a pharmaceutically acceptable carrier or excipient. Such a carrier includes but is not limited to saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The formulation should suit the mode of administration.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. In addition, the soluble form of the receptor and agonists and antagonists of the present invention may also be employed in conjunction with other therapeutic compounds.

The pharmaceutical compositions may be administered in a convenient manner such as by the oral, topical, intravenous, intraperitoneal, intramuscular, subcutaneous, intranasal or intradermal routes. The pharmaceutical compositions are administered in an amount which is effective for treating and/or prophylaxis of the specific indication. In general, they are administered in an amount of at least about 10  $\mu$ g/kg body weight and in most cases they will be administered in an amount not in excess of about 8 mg/Kg body weight per day. In most cases, the dosage is from about 10

$\mu\text{g/kg}$  to about 1 mg/kg body weight daily, taking into account the routes of administration, symptoms, etc.

The TNF receptor and agonists and antagonists which are polypeptides may also be employed in accordance with the present invention by expression of such polypeptides in vivo, which is often referred to as "gene therapy."

Thus, for example, cells from a patient may be engineered with a polynucleotide (DNA or RNA) encoding a polypeptide *ex vivo*, with the engineered cells then being provided to a patient to be treated with the polypeptide. Such methods are well-known in the art. For example, cells may be engineered by procedures known in the art by use of a retroviral particle containing RNA encoding a polypeptide of the present invention.

Similarly, cells may be engineered in vivo for expression of a polypeptide in vivo by, for example, procedures known in the art. As known in the art, a producer cell for producing a retroviral particle containing RNA encoding the polypeptide of the present invention may be administered to a patient for engineering cells in vivo and expression of the polypeptide in vivo. These and other methods for administering a polypeptide of the present invention by such method should be apparent to those skilled in the art from the teachings of the present invention. For example, the expression vehicle for engineering cells may be other than a retrovirus, for example, an adenovirus which may be used to engineer cells in vivo after combination with a suitable delivery vehicle.

Retroviruses from which the retroviral plasmid vectors hereinabove mentioned may be derived include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, retroviruses such as Rous Sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, gibbon ape leukemia virus, human immunodeficiency virus, adenovirus, Myeloproliferative Sarcoma Virus, and mammary tumor virus.



In one embodiment, the retroviral plasmid vector is derived from Moloney Murine Leukemia Virus.

The vector includes one or more promoters. Suitable promoters which may be employed include, but are not limited to, the retroviral LTR; the SV40 promoter; and the human cytomegalovirus (CMV) promoter described in Miller, et al., Biotechniques, Vol. 7, No. 9, 980-990 (1989), or any other promoter (e.g., cellular promoters such as eukaryotic cellular promoters including, but not limited to, the histone, pol III, and  $\beta$ -actin promoters). Other viral promoters which may be employed include, but are not limited to, adenovirus promoters, thymidine kinase (TK) promoters, and B19 parvovirus promoters. The selection of a suitable promoter will be apparent to those skilled in the art from the teachings contained herein.

The nucleic acid sequence encoding the polypeptide of the present invention is under the control of a suitable promoter. Suitable promoters which may be employed include, but are not limited to, adenoviral promoters, such as the adenoviral major late promoter; or heterologous promoters, such as the cytomegalovirus (CMV) promoter; the respiratory syncytial virus (RSV) promoter; inducible promoters, such as the MMT promoter, the metallothionein promoter; heat shock promoters; the albumin promoter; the ApoAI promoter; human globin promoters; viral thymidine kinase promoters, such as the Herpes Simplex thymidine kinase promoter; retroviral LTRs (including the modified retroviral LTRs hereinabove described); the  $\beta$ -actin promoter; and human growth hormone promoters. The promoter also may be the native promoter which controls the gene encoding the polypeptide.

The retroviral plasmid vector is employed to transduce packaging cell lines to form producer cell lines. Examples of packaging cells which may be transfected include, but are not limited to, the PE501, PA317,  $\psi$ -2,  $\psi$ -AM, PA12, T19-14X, VT-19-17-H2,  $\psi$ CRE,  $\psi$ CRIP, GP+E-86, GP+envAm12, and DAN cell

lines as described in Miller, Human Gene Therapy, Vol. 1, pgs. 5-14 (1990), which is incorporated herein by reference in its entirety. The vector may transduce the packaging cells through any means known in the art. Such means include, but are not limited to, electroporation, the use of liposomes, and  $\text{CaPO}_4$  precipitation. In one alternative, the retroviral plasmid vector may be encapsulated into a liposome, or coupled to a lipid, and then administered to a host.

The producer cell line generates infectious retroviral vector particles which include the nucleic acid sequence(s) encoding the polypeptides. Such retroviral vector particles then may be employed, to transduce eukaryotic cells, either in vitro or in vivo. The transduced eukaryotic cells will express the nucleic acid sequence(s) encoding the polypeptide. Eukaryotic cells which may be transduced include, but are not limited to, embryonic stem cells, embryonic carcinoma cells, as well as hematopoietic stem cells, hepatocytes, fibroblasts, myoblasts, keratinocytes, endothelial cells, and bronchial epithelial cells. The sequences of the present invention are also valuable for chromosome identification. The sequence is specifically targeted to and can hybridize with a particular location on an individual human chromosome. Moreover, there is a current need for identifying particular sites on the chromosome. Few chromosome marking reagents based on actual sequence data (repeat polymorphisms) are presently available for marking chromosomal location. The mapping of DNAs to chromosomes according to the present invention is an important first step in correlating those sequences with genes associated with disease.

Briefly, sequences can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp) from the cDNA. Computer analysis of the 3' untranslated region is used to rapidly select primers that do not span more than one exon in

the genomic DNA, thus complicating the amplification process. These primers are then used for PCR screening of somatic cell hybrids containing individual human chromosomes. Only those hybrids containing the human gene corresponding to the primer will yield an amplified fragment.

PCR mapping of somatic cell hybrids is a rapid procedure for assigning a particular DNA to a particular chromosome. Using the present invention with the same oligonucleotide primers, sublocalization can be achieved with panels of fragments from specific chromosomes or pools of large genomic clones in an analogous manner. Other mapping strategies that can similarly be used to map to its chromosome include *in situ* hybridization, prescreening with labeled flow-sorted chromosomes and preselection by hybridization to construct chromosome specific-cDNA libraries.

Fluorescence *in situ* hybridization (FISH) of a cDNA clone to a metaphase chromosomal spread can be used to provide a precise chromosomal location in one step. This technique can be used with cDNA as short as 50 or 60 bases. For a review of this technique, see Verma et al., *Human Chromosomes: a Manual of Basic Techniques*, Pergamon Press, New York (1988).

Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. Such data are found, for example, in V. McKusick, *Mendelian Inheritance in Man* (available on line through Johns Hopkins University Welch Medical Library). The relationship between genes and diseases that have been mapped to the same chromosomal region are then identified through linkage analysis (coinheritance of physically adjacent genes).

Next, it is necessary to determine the differences in the cDNA or genomic sequence between affected and unaffected individuals. If a mutation is observed in some or all of the affected individuals but not in any normal individuals, then

the mutation is likely to be the causative agent of the disease.

With current resolution of physical mapping and genetic mapping techniques, a cDNA precisely localized to a chromosomal region associated with the disease could be one of between 50 and 500 potential causative genes. (This assumes 1 megabase mapping resolution and one gene per 20 kb).

The polypeptides, their fragments or other derivatives, or analogs thereof, or cells expressing them can be used as an immunogen to produce antibodies thereto. These antibodies can be, for example, polyclonal or monoclonal antibodies. The present invention also includes chimeric, single chain, and humanized antibodies, as well as Fab fragments, or the product of an Fab expression library. Various procedures known in the art may be used for the production of such antibodies and fragments.

Antibodies generated against the polypeptides corresponding to a sequence of the present invention can be obtained by direct injection of the polypeptides into an animal or by administering the polypeptides to an animal, preferably a nonhuman. The antibody so obtained will then bind the polypeptides itself. In this manner, even a sequence encoding only a fragment of the polypeptides can be used to generate antibodies binding the whole native polypeptides. Such antibodies can then be used to isolate the polypeptide from tissue expressing that polypeptide.

For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler and Milstein, 1975, Nature, 256:495-497), the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies

(Cole, et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96).

Techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce single chain antibodies to immunogenic polypeptide products of this invention. Also, transgenic mice may be used to express humanized antibodies to immunogenic polypeptide products of this invention.

The present invention will be further described with reference to the following examples; however, it is to be understood that the present invention is not limited to such examples. All parts or amounts, unless otherwise specified, are by weight.

In order to facilitate understanding of the following examples certain frequently occurring methods and/or terms will be described.

"Plasmids" are designated by a lower case p preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are either commercially available, publicly available on an unrestricted basis, or can be constructed from available plasmids in accord with published procedures. In addition, equivalent plasmids to those described are known in the art and will be apparent to the ordinarily skilled artisan.

"Digestion" of DNA refers to catalytic cleavage of the DNA with a restriction enzyme that acts only at certain sequences in the DNA. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors and other requirements were used as would be known to the ordinarily skilled artisan. For analytical purposes, typically 1  $\mu$ g of plasmid or DNA fragment is used with about 2 units of enzyme in about 20  $\mu$ l of buffer solution. For the purpose of isolating DNA fragments for plasmid construction, typically 5 to 50  $\mu$ g of DNA are digested with 20 to 250 units of enzyme in a larger

volume. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation times of about 1 hour at 37°C are ordinarily used, but may vary in accordance with the supplier's instructions. After digestion the reaction is electrophoresed directly on a polyacrylamide gel to isolate the desired fragment.

Size separation of the cleaved fragments is performed using 8 percent polyacrylamide gel described by Goeddel, D. et al., *Nucleic Acids Res.*, 8:4057 (1980).

"Oligonucleotides" refers to either a single stranded polydeoxynucleotide or two complementary polydeoxynucleotide strands which may be chemically synthesized. Such synthetic oligonucleotides have no 5' phosphate and thus will not ligate to another oligonucleotide without adding a phosphate with an ATP in the presence of a kinase. A synthetic oligonucleotide will ligate to a fragment that has not been dephosphorylated.

"Ligation" refers to the process of forming phosphodiester bonds between two double stranded nucleic acid fragments (Maniatis, T., et al., *Id.*, p. 146). Unless otherwise provided, ligation may be accomplished using known buffers and conditions with 10 units of T4 DNA ligase ("ligase") per 0.5 µg of approximately equimolar amounts of the DNA fragments to be ligated.

Unless otherwise stated, transformation was performed as described in the method of Graham, F. and Van der Eb, A., *Virology*, 52:456-457 (1973).

#### Example 1

##### Bacterial Expression and Purification of the TNF receptor

The DNA sequence encoding TNF receptor, ATCC # 75899, is initially amplified using PCR oligonucleotide primers corresponding to the 5' and 3' end sequences of the processed TNF receptor nucleic acid sequence (minus the signal peptide sequence). Additional nucleotides corresponding to TNF

receptor gene are added to the 5' and 3' end sequences respectively. The 5' oligonucleotide primer has the sequence 5' GCCAGAGGATCCGAAACGTTTCCTCCAAAGTAC 3' (SEQ ID No. 3) contains a BamHI restriction enzyme site (bold) followed by 21 nucleotides of TNF receptor coding sequence starting from the presumed initiation codon. The 3' sequence 5' CGGCTTCTAGAATTACCTATCATTTCTAAAAAT 3' (SEQ ID No. 4) contains complementary sequences to a Hind III site (bold) and is followed by 18 nucleotides of TNF receptor. The restriction enzyme sites correspond to the restriction enzyme sites on the bacterial expression vector pQE-9 (Qiagen, Inc. Chatsworth, CA). pQE-9 encodes antibiotic resistance (Amp<sup>r</sup>), a bacterial origin of replication (ori), an IPTG-regulatable promoter operator (P/O), a ribosome binding site (RBS), a 6-His tag and restriction enzyme sites. pQE-9 is then digested with BamHI and XbaI. The amplified sequences are ligated into pQE-9 and are inserted in frame with the sequence encoding for the histidine tag and the RBS. The ligation mixture is then used to transform *E. coli* strain M15/rep 4 (Qiagen, Inc.) by the procedure described in Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, Cold Spring Laboratory Press, (1989). M15/rep4 contains multiple copies of the plasmid pREP4, which expresses the lacI repressor and also confers kanamycin resistance (Kan<sup>r</sup>). Transformants are identified by their ability to grow on LB plates and ampicillin/kanamycin resistant colonies are selected. Plasmid DNA is isolated and confirmed by restriction analysis. Clones containing the desired constructs are grown overnight (O/N) in liquid culture in LB media supplemented with both Amp (100 ug/ml) and Kan (25 ug/ml). The O/N culture is used to inoculate a large culture at a ratio of 1:100 to 1:250. The cells are grown to an optical density 600 (O.D.<sup>600</sup>) of between 0.4 and 0.6. IPTG ("Isopropyl-B-D-thiogalacto pyranoside") is then added to a final concentration of 1 mM. IPTG induces by inactivating the lacI

repressor, clearing the P/O leading to increased gene expression. Cells are grown an extra 3 to 4 hours. Cells are then harvested by centrifugation. The cell pellet is solubilized in the chaotropic agent 6 Molar Guanidine HCl. After clarification, solubilized TNF receptor is purified from this solution by chromatography on a Nickel-Chelate column under conditions that allow for tight binding by proteins containing the 6-His tag (Hochuli, E. et al., J. Chromatography 411:177-184 (1984)). TNF receptor (90% pure) is eluted from the column in 6 molar guanidine HCl pH 5.0 and for the purpose of renaturation adjusted to 3 molar guanidine HCl, 100mM sodium phosphate, 10 mmolar glutathione (reduced) and 2 mmolar glutathione (oxidized). After incubation in this solution for 12 hours the protein is dialyzed to 10 mmolar sodium phosphate.

#### Example 2

##### Cloning and expression of TNF receptor and extracellular (soluble) TNF receptor using the baculovirus expression system

The DNA sequence encoding the full length TNF receptor protein, ATCC # 75899, was amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the gene. The 5' primer has the sequence 5' GCGCGGATCC**ATGAACAAGTTGCTGTGCTGC** 3' (SEQ ID No. 5) and contains a BamHI restriction enzyme site (in bold) and which is just behind the first 21 nucleotides of the TNF receptor gene (the initiation codon for translation "ATG" is underlined).

The 3' primer has the sequence 5' GCGCTCTAGATTA CCTATCATTCTAAAAATAAC 3' (SEQ ID No. 6) and 5' GCGCGGTACCTCAGTGGTTTGGGCTCCTCCC 3' (SEQ ID No. 7) and contains the cleavage site for the restriction endonuclease XbaI and 21 nucleotides complementary to the 3' non-translated sequence of the TNF receptor gene. The amplified



sequences were isolated from a 1% agarose gel using a commercially available kit ("Geneclean", BIO 101 Inc., La Jolla, Ca.). The fragments were then digested with the endonucleases BamHI and XbaI and then purified again on a 1% agarose gel. This fragment is designated F2.

The vector pRG1 (modification of pVL941 vector, discussed below) was used for the expression of the TNF receptor proteins using the baculovirus expression system (for review see: Summers, M.D. and Smith, G.E. 1987, A manual of methods for baculovirus vectors and insect cell culture procedures, Texas Agricultural Experimental Station Bulletin No. 1555). This expression vector contains the strong polyhedrin promoter of the Autographa californica nuclear polyhedrosis virus (AcMNPV) followed by the recognition sites for the restriction endonucleases BamHI and XbaI. The polyadenylation site of the simian virus (SV)40 was used for efficient polyadenylation. For an easy selection of recombinant viruses the beta-galactosidase gene from E.coli was inserted in the same orientation as the polyhedrin promoter followed by the polyadenylation signal of the polyhedrin gene. The polyhedrin sequences were flanked at both sides by viral sequences for the cell-mediated homologous recombination of cotransfected wild-type viral DNA. Many other baculovirus vectors could be used in place of pRG1 such as pAc373, pVL941 and pAcIM1 (Luckow, V.A. and Summers, M.D., Virology, 170:31-39).

The plasmid was digested with the restriction enzymes BamHI and XbaI. The DNA was then isolated from a 1% agarose gel using the commercially available kit ("Geneclean" BIO 101 Inc., La Jolla, Ca.). This vector DNA is designated V2.

Fragment F2 and the dephosphorylated plasmid V2 were ligated with T4 DNA ligase. E. coli HB101 cells were then transformed and cells identified that contained the plasmid (pBac TNF receptor) with the TNF receptor genes using the

enzymes BamHI and XbaI. The sequence of the cloned fragment was confirmed by DNA sequencing.

5  $\mu$ g of the plasmid pBac TNF receptor was cotransfected with 1.0  $\mu$ g of a commercially available linearized baculovirus ("BaculoGold" baculovirus DNA", Pharmingen, San Diego, CA.) using the lipofection method (Felgner et al. Proc. Natl. Acad. Sci. USA, 84:7413-7417 (1987)).

1  $\mu$ g of BaculoGold<sup>™</sup> virus DNA and 5  $\mu$ g of the plasmid pBac TNF receptors were mixed in a sterile well of a microtiter plate containing 50  $\mu$ l of serum free Grace's medium (Life Technologies Inc., Gaithersburg, MD). Afterwards 10  $\mu$ l Lipofectin plus 90  $\mu$ l Grace's medium were added, mixed and incubated for 15 minutes at room temperature. Then the transfection mixture was added dropwise to the Sf9 insect cells (ATCC CRL 1711) seeded in a 35 mm tissue culture plate with 1ml Grace' medium without serum. The plate was rocked back and forth to mix the newly added solution. The plate was then incubated for 5 hours at 27°C. After 5 hours the transfection solution was removed from the plate and 1 ml of Grace's insect medium supplemented with 10% fetal calf serum was added. The plate was put back into an incubator and cultivation continued at 27°C for four days.

After four days the supernatant was collected and a plaque assay performed similar as described by Summers and Smith (supra). As a modification an agarose gel with "Blue Gal" (Life Technologies Inc., Gaithersburg) was used which allows an easy isolation of blue stained plaques. (A detailed description of a "plaque assay" can also be found in the user's guide for insect cell culture and baculovirology distributed by Life Technologies Inc., Gaithersburg, page 9-10).

Four days after the serial dilution, the viruses were added to the cells and blue stained plaques were picked with the tip of an Eppendorf pipette. The agar containing the

recombinant viruses were then resuspended in an Eppendorf tube containing 200  $\mu$ l of Grace's medium. The agar was removed by a brief centrifugation and the supernatant containing the recombinant baculoviruses was used to infect Sf9 cells seeded in 35 mm dishes. Four days later the supernatants of these culture dishes were harvested and then stored at 4°C.

Sf9 cells were grown in Grace's medium supplemented with 10% heat-inactivated FBS. The cells were infected with the recombinant baculovirus V-TNF receptor at a multiplicity of infection (MOI) of 2. Six hours later the medium was removed and replaced with SF900 II medium minus methionine and cysteine (Life Technologies Inc., Gaithersburg). 42 hours later 5  $\mu$ Ci of  $^{35}$ S-methionine and 5  $\mu$ Ci  $^{35}$ S cysteine (Amersham) were added. The cells are further incubated for 16 hours before they are harvested by centrifugation and the labelled proteins visualized by SDS-PAGE and autoradiography.

### Example 3

#### Expression of Recombinant TNF receptor in COS cells

The expression of plasmid, TNF receptor HA is derived from a vector pcDNAI/Amp (Invitrogen) containing: 1) SV40 origin of replication, 2) ampicillin resistance gene, 3) E.coli replication origin, 4) CMV promoter followed by a polylinker region, a SV40 intron and polyadenylation site. A DNA fragment encoding the entire TNF receptor precursor and a HA tag fused in frame to its 3' end is cloned into the polylinker region of the vector, therefore, the recombinant protein expression is directed under the CMV promoter. The HA tag correspond to an epitope derived from the influenza hemagglutinin protein as previously described (I. Wilson, H. Niman, R. Heighten, A. Cherenson, M. Connolly, and R. Lerner, 1984, Cell 37, 767). The infusion of HA tag to the target protein allows easy detection of the recombinant protein with an antibody that recognizes the HA epitope.

The plasmid construction strategy is described as follows:

The DNA sequence encoding TNF receptor, ATCC # 75899, is constructed by PCR using two primers: the 5' primer 5' GCCAGAGGATCCGCCACCATGAACAAGTTGCTGTGCTGC 3' (SEQ ID No. 8) contains a BamHI site (bold) followed by 21 nucleotides of TNF receptor coding sequence starting from the initiation codon; the 3' sequence 5' CGGCTTCTAGAATCAAGCGTAGTCTGGGACG TCGTATGGGTACCTATCATTCTAAAAAT 3' (SEQ ID No. 9) contains complementary sequences to an XbaI site (bold), translation stop codon, HA tag and the last 18 nucleotides of the TNF receptor coding sequence (not including the stop codon). Therefore, the PCR product contains a BamHI site, TNF receptor coding sequence followed by HA tag fused in frame, a translation termination stop codon next to the HA tag, and an XbaI site. The PCR amplified DNA fragment and the vector, pCDNAI/Amp, are digested with BamHI and XbaI restriction enzymes and ligated. The ligation mixture is transformed into E. coli strain SURE (Stratagene Cloning Systems, La Jolla, CA) the transformed culture is plated on ampicillin media plates and resistant colonies are selected. Plasmid DNA is isolated from transformants and examined by restriction analysis for the presence of the correct fragment. For expression of the recombinant TNF receptor, COS cells are transfected with the expression vector by DEAE-DEXTRAN method (J. Sambrook, E. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Laboratory Press, (1989)). The expression of the TNF receptor HA protein is detected by radiolabelling and immunoprecipitation method (E. Harlow, D. Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, (1988)). Cells are labelled for 8 hours with <sup>35</sup>S-cysteine two days post transfection. Culture media are then collected and cells are lysed with detergent (RIPA buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, 1% NP-40, 0.5% DOC, 50mM Tris, pH 7.5) (Wilson, I. et al., Id. 37:767 (1984)).

Both cell lysate and culture media are precipitated with a HA specific monoclonal antibody. Proteins precipitated are analyzed on 15% SDS-PAGE gels.

#### Example 4

##### Expression via Gene Therapy

Fibroblasts are obtained from a subject by skin biopsy. The resulting tissue is placed in tissue-culture medium and separated into small pieces. Small chunks of the tissue are placed on a wet surface of a tissue culture flask, approximately ten pieces are placed in each flask. The flask is turned upside down, closed tight and left at room temperature over night. After 24 hours at room temperature, the flask is inverted and the chunks of tissue remain fixed to the bottom of the flask and fresh media (e.g., Ham's F12 media, with 10% FBS, penicillin and streptomycin, is added. This is then incubated at 37°C for approximately one week. At this time, fresh media is added and subsequently changed every several days. After an additional two weeks in culture, a monolayer of fibroblasts emerge. The monolayer is trypsinized and scaled into larger flasks.

pMV-7 (Kirschmeier, P.T. et al, DNA, 7:219-25 (1988) flanked by the long terminal repeats of the Moloney murine sarcoma virus, is digested with EcoRI and HindIII and subsequently treated with calf intestinal phosphatase. The linear vector is fractionated on agarose gel and purified, using glass beads.

The cDNA encoding a polypeptide of the present invention is amplified using PCR primers which correspond to the 5' and 3' end sequences respectively. The 5' primer containing an EcoRI site and the 3' primer further includes a HindIII site. Equal quantities of the Moloney murine sarcoma virus linear backbone and the amplified EcoRI and HindIII fragment are added together, in the presence of T4 DNA ligase. The resulting mixture is maintained under conditions appropriate for ligation of the two fragments. The ligation mixture is

used to transform bacteria HB101, which are then plated onto agar-containing kanamycin for the purpose of confirming that the vector had the gene of interest properly inserted.

The amphotropic pA317 or GP+aml2 packaging cells are grown in tissue culture to confluent density in Dulbecco's Modified Eagles Medium (DMEM) with 10% calf serum (CS), penicillin and streptomycin. The MSV vector containing the gene is then added to the media and the packaging cells are transduced with the vector. The packaging cells now produce infectious viral particles containing the gene (the packaging cells are now referred to as producer cells).

Fresh media is added to the transduced producer cells, and subsequently, the media is harvested from a 10 cm plate of confluent producer cells. The spent media, containing the infectious viral particles, is filtered through a millipore filter to remove detached producer cells and this media is then used to infect fibroblast cells. Media is removed from a sub-confluent plate of fibroblasts and quickly replaced with the media from the producer cells. This media is removed and replaced with fresh media. If the titer of virus is high, then virtually all fibroblasts will be infected and no selection is required. If the titer is very low, then it is necessary to use a retroviral vector that has a selectable marker, such as neo or his.

The engineered fibroblasts are then injected into the host, either alone or after having been grown to confluence on cytodex 3 microcarrier beads. The fibroblasts now produce the protein product. Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, within the scope of the appended claims, the invention may be practiced otherwise than as particularly described.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: GREENE, ET AL.

(ii) TITLE OF INVENTION: Human Tumor Necrosis Factor  
Receptor

(iii) NUMBER OF SEQUENCES: 9

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: CARELLA, BYRNE, BAIN, GILFILLAN,  
CECCHI, STEWART & OLSTEIN

(B) STREET: 6 BECKER FARM ROAD

(C) CITY: ROSELAND

(D) STATE: NEW JERSEY

(E) COUNTRY: USA

(F) ZIP: 07068

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3.5 INCH DISKETTE

(B) COMPUTER: IBM PS/2

(C) OPERATING SYSTEM: MS-DOS

(D) SOFTWARE: WORD PERFECT 5.1

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE: Concurrently

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA

(A) APPLICATION NUMBER: PCT/US95/03216

(B) FILING DATE: 15 MAR 1995

(viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: FERRARO, GREGORY D.  
(B) REGISTRATION NUMBER: 36,134  
(C) REFERENCE/DOCKET NUMBER: 325800-381

(ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: 201-994-1700  
(B) TELEFAX: 201-994-1744

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 1173 BASE PAIRS  
(B) TYPE: NUCLEIC ACID  
(C) STRANDEDNESS: SINGLE  
(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
ATGAACAAGT TGCTGTGCTG CGCGCTCGTG TTTCTGGACA TCTCCATTAA GTGGACCACC 60
CAGGAAACGT TTCCTCCAAA GTACCTTCAT TATGACGAAG AAACCTCTCA TCAGCTGTTG 120
TGTGACAAAT GTCCTCCTGG TACCTACCTA AAACAACACT GTACAGCAAA GTGGAGGACC 180
GTGTGCGCCC CTTGCCCTGA CCACTACTAC ACAGACAGCT GGCACACCA3 TGACGAGTGT 240
CTATACTGCA GCCCCGTGTG CAAGGAGCTG CAGTACGTCA AGCAGGAGTG CAATCGCACC 300
CACAACCGCG TGTGCGAATG CAAGGAAGGG CGCTACCTTG AGATAGAGTT CTGCTTGAAA 360
CATAGGAGCT GCCCTCCTGG ATTTGGAGTG GTGCAAGCTG GAACCCCAAG GCGAATACA 420
GTTTGCAAAA GATGTCCAGA TGGGTTCTTC TCAAATGAGA CGTCATCTAA AGCACCCGT 480
AGAAAACACA CAAATTGCAG TGTCTTTGGT CTCTGCTAA CTCAGAAAGG AAATGCAACA 540
CACGACAACA TATGTTCCGG AAACAGTGAA TCAACTCAA AATGTGGAAT AGATGTTACC 600
CTGTGTGAGG AGGCATTCTT CAGGTTTGCT GTTCTTACAA AGTTTACGCC TAACTGGCTT 660
AGTGTCTTGG TAGACAATTT GCCTGGCACC AAAGTAAACG CAGAGAGTGT AGAGAGGATA 720
AAACGGCAAC ACAGCTCACA AGAACAGACT TTCCAGCTGC TGAAGTTATG GAAACATCAA 780
AACAAAGACC AAGATATAGT CAAGAAGATC ATCCAAGATA TTGACCTCTG TGAACACAGC 840
GTGCAGCGGC ACATTGGACA TGCTAACCTC ACCTTCGAGC AGCTTCGTAG CTTGATGGAA 900
AGCTTACCGG GAAAGAAAGT GGGAGCAGAA GACATTGAAA AAACAATAAA GGCATGCAAA 960
CCCAGTGACC AGATCTGAA GCTGCTCAGT TTGTGGCGAA TAAAAAATGG CGACCAAGAC 1020
```



ACCTTGAAGG GCTTAATGCA CGCACTAAAG CACTCAAAGA CGTACCATT TCCCACAAAC 1080  
 TCTCACTCAG AGTCTAAAGA AGACCATCAG GTTCCTTCAC AGCTTCACAA TGTACAAATT 1140  
 GTATCAGAAG TTATTTTITAG AAATGATAGG TAA 1173

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 390 AMINO ACIDS
- (B) TYPE: AMINO ACID
- (C) STRANDEDNESS:
- (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: PROTEIN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met	Asn	Lys	Leu	Leu	Cys	Cys	Ala	Leu	Val	Phe	Leu	Asp	Ile	Ser
-20					-15					-10				
Ile	Lys	Trp	Thr	Thr	Gln	Glu	Thr	Phe	Pro	Pro	Lys	Tyr	Leu	His
-5					1					5				
Tyr	Asp	Glu	Glu	Thr	Ser	His	Gln	Leu	Leu	Cys	Asp	Lys	Cys	Pro
10					15					20				
Pro	Gly	Thr	Tyr	Leu	Lys	Gln	His	Cys	Thr	Ala	Lys	Trp	Lys	Thr
25					30					35				
Val	Cys	Ala	Pro	Cys	Pro	Asp	His	Tyr	Tyr	Thr	Asp	Ser	Trp	His
40					45					50				
Thr	Ser	Asp	Glu	Cys	Leu	Tyr	Cys	Ser	Pro	Val	Cys	Lys	Glu	Leu
55					60					65				
Gln	Tyr	Val	Lys	Gln	Glu	Cys	Asn	Arg	Thr	His	Asn	Arg	Val	Cys
70					75					80				
Glu	Cys	Lys	Glu	Gly	Arg	Tyr	Leu	Glu	Ile	Glu	Phe	Cys	Leu	Lys
85					90					95				
His	Arg	Ser	Cys	Pro	Pro	Gly	Phe	Gly	Val	Val	Gln	Ala	Gly	Thr
100					105					110				
Pro	Glu	Arg	Asn	Thr	Val	Cys	Lys	Arg	Cys	Pro	Asp	Gly	Phe	Phe
115					120					125				

Ser Asn Glu Thr Ser Ser Lys Ala Pro Cys Arg Lys His Thr Asn		
130	135	140
Cys Ser Val Phe Gly Leu Leu Leu Thr Gln Lys Gly Asn Ala Thr		
145	150	155
His Asp Asn Ile Cys Ser Gly Asn Ser Glu Ser Thr Gln Lys Cys		
160	165	170
Gly Ile Asp Val Thr Leu Cys Glu Glu Ala Phe Phe Arg Phe Ala		
175	180	185
Val Pro Thr Lys Phe Thr Pro Asn Trp Leu Ser Val Leu Val Asp		
190	195	200
Asn Leu Pro Gly Thr Lys Val Asn Ala Glu Ser Val Glu Arg Ile		
205	210	215
Lys Arg Gln His Ser Ser Gln Glu Gln Thr Phe Gln Leu Leu Lys		
220	225	230
Leu Trp Lys His Gln Asn Lys Asp Gln Asp Ile Val Lys Lys Ile		
235	240	245
Ile Gln Asp Ile Asp Leu Cys Glu Asn Ser Val Gln Arg His Ile		
250	255	260
Gly His Ala Asn Leu Thr Phe Glu Gln Leu Arg Ser Leu Met Glu		
265	270	275
Ser Leu Pro Gly Lys Lys Val Gly Ala Glu Asp Ile Glu Lys Thr		
280	285	290
Ile Lys Ala Cys Lys Pro Ser Asp Gln Ile Leu Lys Leu Leu Ser		
295	300	305
Leu Trp Arg Ile Lys Asn Gly Asp Gln Asp Thr Leu Lys Gly Leu		
310	315	320
Met His Ala Leu Lys His Ser Lys Thr Tyr His Phe Pro Thr Asn		
325	330	335
Cys His Ser Glu Ser Lys Glu Asp His Gln Val Pro Ser Gln Leu		
340	345	350
His Asn Val Gln Ile Val Ser Glu Val Ile Phe Arg Asn Asp Arg		
355	360	365

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS  
(A) LENGTH: 33 BASE PAIRS  
(B) TYPE: NUCLEIC ACID  
(C) STRANDEDNESS: SINGLE  
(D) TOPOLOGY: LINEAR
- (ii) MOLECULE TYPE: Oligonucleotide
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GCCAGAGGAT CCGAAACGTT TCCTCAAAG TAC

33

- (2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS  
(A) LENGTH: 33 BASE PAIRS  
(B) TYPE: NUCLEIC ACID  
(C) STRANDEDNESS: SINGLE  
(D) TOPOLOGY: LINEAR
- (ii) MOLECULE TYPE: Oligonucleotide
- (xi) SEQUENCE DESCRIPTION: SEQ ID NC:4:

CGGCTTCTAG AATTACCTAT CATTCTAAA AAT

33

- (2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS  
(A) LENGTH: 31 BASE PAIRS  
(B) TYPE: NUCLEIC ACID  
(C) STRANDEDNESS: SINGLE  
(D) TOPOLOGY: LINEAR
- (ii) MOLECULE TYPE: Oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCGCGGATCC ATGAACAAGT TGCTGTGCTG C

31

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 34 BASE PAIRS

(B) TYPE: NUCLEIC ACID

(C) STRANDEDNESS: SINGLE

(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: Oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCGCTCTAGA TTACCTATCA TTTCTAAAAA TAAC

34

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 31 BASE PAIRS

(B) TYPE: NUCLEIC ACID

(C) STRANDEDNESS: SINGLE

(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: Oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCGCGGTACC TCAGTGGTTT GGGCTCCTCC C

31

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 39 BASE PAIRS
- (B) TYPE: NUCLEIC ACID
- (C) STRANDEDNESS: SINGLE
- (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: Oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GCCAGAGGAT CCGCCACCAT GAACAAGTTG CTGTGCTGC

39

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 60 BASE PAIRS
- (B) TYPE: NUCLEIC ACID
- (C) STRANDEDNESS: SINGLE
- (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: Oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CGGCTTCTAG AATCAAGCGT AGTCTGGGAC GTCGTATGGG TACCTATCAT TTCTAAAAAT

60

WHAT IS CLAIMED IS:

1. An isolated polynucleotide comprising a member selected from the group consisting of:
  - (a) a polynucleotide encoding the polypeptide comprising amino acid -21 to 369 as set forth in SEQ ID NO:2;
  - (b) a polynucleotide encoding the polypeptide comprising amino acid 1 to 369 as set forth in SEQ ID NO:2;
  - (c) a polynucleotide capable of hybridizing to and which is at least 70% identical to the polynucleotide of (a) or (b); and
  - (d) a polynucleotide fragment of the polynucleotide of (a), (b) or (c).
2. The polynucleotide of Claim 1 wherein the polynucleotide is DNA.
3. An isolated polynucleotide comprising a member selected from the group consisting of:
  - (a) a polynucleotide encoding a mature polypeptide encoded by the DNA contained in ATCC Deposit No. 75899;
  - (b) a polynucleotide encoding a polypeptide expressed by the DNA contained in ATCC Deposit No. 75899;
  - (c) a polynucleotide capable of hybridizing to and which is at least 70% identical to the polynucleotide of (a) or (b); and
  - (d) a polynucleotide fragment of the polynucleotide of (a), (b) or (c).
4. A vector containing the DNA of Claim 2.
5. A host cell transformed or transfected with the vector of Claim 4.

6. A process for producing a polypeptide comprising: expressing from the host cell of Claim 5 the polypeptide encoded by said DNA.

7. A process for producing cells capable of expressing a polypeptide comprising transforming or transfecting the cells with the vector of Claim 4.

8. A receptor polypeptide comprising a member selected from the group consisting of:

(i) a polypeptide having the deduced amino acid sequence of SEQ ID NO:2 and fragments, analogs and derivatives thereof; and

(ii) a polypeptide encoded by the cDNA of ATCC Deposit No. 75899 and fragments, analogs and derivatives of said polypeptide.

9. An antibody against the polypeptide of claim 8.

10. A compound which activates the polypeptide of claim 8.

11. A compound which inhibits activation the polypeptide of claim 8.

12. A method for the treatment of a patient having need to activate a TNF receptor comprising: administering to the patient a therapeutically effective amount of the compound of claim 10.

13. A method for the treatment of a patient having need to inhibit a TNF receptor comprising: administering to the patient a therapeutically effective amount of the compound of claim 11.

14. The method of claim 12 wherein said compound is a polypeptide and a therapeutically effective amount of the compound is administered by providing to the patient DNA encoding said agonist and expressing said agonist *in vivo*.

15. The method of claim 13 wherein said compound is a polypeptide and a therapeutically effective amount of the compound is administered by providing to the patient DNA encoding said antagonist and expressing said antagonist *in vivo*.

16. A method for identifying compounds which bind to and activate the receptor polypeptide of claim 8 comprising:

contacting a cell expressing on the surface thereof the receptor polypeptide, said receptor being associated with a second component capable of providing a detectable signal in response to the binding of a compound to said receptor polypeptide, with a compound under conditions sufficient to permit binding of the compound to the receptor polypeptide; and

identifying if the compound is capable of receptor binding by detecting the signal produced by said second component.

17. A method for identifying compounds which bind to and inhibit activation of the polypeptide of claim 8 comprising:

contacting a cell expressing on the surface thereof the receptor polypeptide, said receptor being associated with a second component capable of providing a detectable signal in response to the binding of a compound to said receptor polypeptide, with an analytically detectable ligand known to bind to the receptor polypeptide



and a compound to be screened under conditions to permit binding to the receptor polypeptide; and

determining whether the compound inhibits activation of the polypeptide by detecting the absence of a signal generated from the interaction of the ligand with the polypeptide.

18. A process for diagnosing a disease or a susceptibility to a disease related to an under-expression of the polypeptide of claim 8 comprising:

determining a mutation in the nucleic acid sequence encoding said polypeptide.

19. The polypeptide of Claim 8 wherein the polypeptide is a soluble fragment of the polypeptide and is capable of binding a ligand for the receptor.

20. A diagnostic process comprising:  
analyzing for the presence of the polypeptide of claim 19 in a sample derived from a host.



# ABSTRACT OF THE DISCLOSURE

A receptor and DNA (RNA) encoding such receptor and a procedure for producing such receptor by recombinant techniques is disclosed. Also disclosed are methods for utilizing such receptor for screening for antagonists and agonists to the receptor and for ligands for the receptor. Also disclosed are methods for utilizing such agonists to inhibit the growth of tumors, to stimulate cellular differentiation, to mediate the immune response and anti-viral response, to regulate growth and provide resistance to certain infections. The use of the antagonists as a therapeutic to treat autoimmune diseases, inflammation, septic shock, to inhibit graft-host reactions, and to prevent apoptosis is also disclosed. Also disclosed are diagnostic methods for detecting mutations in the nucleic acid sequence encoding the receptor and for detecting altered levels of the soluble receptor in a sample derived from a host.

**DECLARATION AND POWER OF ATTORNEY**

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

**HUMAN TUMOR NECROSIS FACTOR RECEPTOR**

the specification of which [X] is attached hereto or [ ] was filed on \_\_\_\_\_ as Application Serial No. \_\_\_\_\_ and was amended on \_\_\_\_\_ (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed. Prior Foreign Application(s):

Priority Claimed  
Yes No

(Number) (Country) (Day/Month/Year Filed)

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, Section 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

PCT/US95/03216  
(Application Serial No.)

March 15, 1995  
(Filing Date)

Pending  
(Status - patented, pending, abandoned)

(Application Serial No.)

(Filing Date)

(Status - patented, pending, abandoned)

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: John N. Bain (Reg. No. 18,651); John G. Gilfillan, III (Reg. No. 22,746); Elliot M. Olstein (Reg. No. 24,025); Raymond J. Lillie (Reg. No. 31,778); Charles J. Herron (Reg. No. 28,019); William Squire (Reg. No. 25,378); and Gregory Ferraro (Reg. No. 36,134) of Carella, Byrne, Bain, Gilfillan, Cecchi, Stewart & Olstein; Robert H. Benson (Reg. No. 30,446) of Human Genome Sciences, Inc., 9410 Key West Ave, Rockville, Maryland 20850-3338. Address correspondence and telephone calls to Gregory D. Ferraro c/o Carella, Byrne, Bain, Gilfillan, Cecchi, Stewart & Olstein, 6 Becker Farm Road, Roseland, NJ 07068 - (201) 994-1700.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first inventor: John M. Greene

Inventor's signature: John M. Greene

Date: JUNE 2, 1995

Residence: 872 Diamond Drive, Gaithersburg, Maryland 20878

Citizenship: USA

Post Office Address: Same

Full name of second joint inventor: Robert D. Fleischmann

Inventor's signature: Robert D. Fleischmann

Date: 6/1/95

Residence: 470 Tschiffely Square Road, Gaithersburg, Maryland 20878

Citizenship: USA

Post Office Address: Same

Docket No.: 325800-381

1 / 5

## FIG. 1A

1 ATGAACAAGTTGCTGTGCTGCGCGCTCGTGTTCCTGGACAT  
M N K L L C C A L V F L D I  
42 CTCCATTAAAGTGGACCAACCAGGAAACGTTTCCTCCAAAGT  
S I K W T T Q E T F P P K  
83 ACCTTCATTATGACGAAGAAACCTCTCATCAGCTGTGTGT  
Y L H Y D E E T S H Q L L C  
124 GACAAATGTCCTCCTGGTACCTACCTAAACAACACTGTAC  
D K C P P G T Y L K Q H C T  
165 AGCAAAGTGGAAAGACCGTGTGCGCCCTTGCCCTGACCACT  
A K W K T V C A P C P D H  
206 ACTACACAGACAGCTGGCACACCAGTGACGAGTGTCTATAC  
Y Y T D S W H T S D E C L Y  
247 TGCAGCCCCGTGTGCAAGGAGCTGCAGTACGTCAAGCAGGA  
C S P V C K E L Q Y V K Q E  
288 GTGCAATCGCACCCACAACCGGTGTGCGAATGCAAGGAAG  
C N R T H N R V C E C K E  
329 GCGCTACCTTGAGATAGAGTTCTGCTTGAAACATAGGAGC  
G R Y L E I E F C L K H R S  
370 TGCCCTCCTGGATTGTGGAGTGGTGCAAGCTGGAACCCAGA  
C P P G F G V V Q A G T P E  
411 GCGAAATACAGTTTGCAAAAGATGTCCAGATGGGTTCCTCT  
R N T V C K R C P D G F F  
452 CAAATGAGACGTCATCTAAAGCACCTGTAGAAACACACA  
S N E T S S K A P C R K H T  
493 AATTGCAGTGTCTTTGGTCTCCTGCTAACTCAGAAAGGAAA  
N C S V F G L L L T Q K G N  
534 TGCAACACACGACAACATATGTTCCGGAAACAGTGAATCAA  
A T H D N I C S G N S E S

MATCH WITH FIG. 1B

32500-381

02/469637

2 / 5 MATCH WITH FIG. 1A

575 CTCAAAAATGTGGAATAGATGTTACCCCTGTGTGAGGAGGCA  
T Q K C G I D V T L C E E A  
616 TTCTTCAGGTTTGTCTGTTCCCTACAAAGTTTACGCCTAACTG  
F F R F A V P T K F T P N W  
657 GCTTAGTGICTTGGTAGACAATTTGCCTGGCACCAAAGTAA  
L S V L V D N L P G T K V  
698 ACCGAGAGAGTGTAGAGAGGATAAAACGGCAACACAGCTCA  
N A E S V E R I K R Q H S S  
739 CAAGAACAGACTTTCCAGCTGCTGAAGTTATGGAACATCA  
Q E Q T F Q L L K L W K H Q  
780 AAACAAAGACCAAGATATAGTCAAGAAGATCATCCAAGATA  
N K D Q D I V K K I I Q D  
821 TTGACCTCTGTGAAACAGCGTGCAGCGGCACATTGGACAT  
I D L C E N S V Q R H I G H  
862 GCTAACCTCACCTTCGAGCAGCTTCGTAGCTTGATGGAAG  
A N L T F E Q L R S L M E S  
903 CTTACCGGGAAAGAAAGTGGGAGCAGAAGACATTGAAAAAA  
L P G K K V G A E D I E K  
944 CAATAAAGGCATGCAAACCCAGTGACCAGATCCTGAAGCTG  
T I K A C K P S D Q I L K L  
985 CTCAGTTTGTGGCGAATAAAAAATGGCGACCAAGACACCTT  
L S L W R I K N G D Q D T L  
1026 GAAGGGCCTAATGCACGCACTAAAGCACTCAAAGACGTACC  
K G L M H A L K H S K T Y  
1067 ACTTTCCCAAACTGTCACTCAGAGTCTAAAGAAGACCAT  
H F P T N C H S E S K E D H  
1108 CAGGTTCCCTTCACAGCTTCACAATGTACAAATTGTATCAGA  
Q V P S Q L H N V Q I V S E  
1149 AGTTATTTTITAGAAATGATAGGTAA  
V I F R N D R .

FIG. 1B

325800-36

# FIG. 2A

tnfr2.msf(TNFR2_LIKE)	1	.....MNKLL CCALVFLDIS IKWTTQETFP P.....	50	KYLHYDEETS
tnfr2.msf(TNR2_HUMAN)		MAPVAVWAAL AVGLELWAAA HALPAQVAFT PYAPEPGSTC RLREYVDQTA		-----Y-----T-
Consensus		-----L-----L-----Q-----F-----P-----		
tnfr2.msf(TNFR2_LIKE)	51	HQLLCDKCSP GTYLKQHCTA KWKTVCAPCP DHYYTDSWHT SDECLYCSVP	100	
tnfr2.msf(TNR2_HUMAN)		.QMCCSKCSP GQAKVFC TK TSDTVCDSC DSTYTQLWNW VPECLSCGSR		-----ECL-C-----
Consensus		-Q--C-KC-P G---K-CT- ---TVC--C- D--Y--W--		
tnfr2.msf(TNFR2_LIKE)	101	CKELQYVKQE CNRTHNRVCE CKEGRYLEIE .....FCLK HRSCPPGFGV	150	
tnfr2.msf(TNR2_HUMAN)		CSSDQVETQA CTREQNRICT CRPGWYCAL KQEGCRLCAP LRKCRPGFGV		-----C-----R-C-PGFGV
Consensus		C---Q---Q- C-R--NR-C- C--G-Y-----		
tnfr2.msf(TNFR2_LIKE)	151	VQAGTPERNT VCKRCPDGFF SNETSSKAPC RKHTNCSVFG LLLTQKGNAT	200	
tnfr2.msf(TNR2_HUMAN)		ARPGTETSDV VCKPCAPGTF SNTTSSTDIC RPHQICNVVA I....PGNAS		-----GNA-
Consensus		---GT-----VCK-C--G-F SN-TSS---C R-H--C-V--		
tnfr2.msf(TNFR2_LIKE)	201	HDNIC.....SGNSE STQKCGIDVT LCEEAF...	250	
tnfr2.msf(TNR2_HUMAN)		RDAVCTSTSP TRSMAPGAVH LPQPVSTRSQ HTQPTPEPST APSTSFLLPM		-----T-----F-----
Consensus		-D--C-----S--S--TQ-----T-----		

MATCH WITH FIG. 2B

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## FIG. 2B

MATCH WITH FIG. 2A

251 300  
 tnfr2.msf(TNFR2\_LIKE) .RFAVPTKFT PNWLSVLVDN LPGTKVNAES VERIKR....  
 tnfr2.msf(TNFR2\_HUMAN) GPSPPAEGST GDFALPVGLI VG..VTALGL LIIGVNCVI MTQVKKKPLC  
 Consensus ----- --FA-P----- L-----VN-----K-----

301 350  
 tnfr2.msf(TNFR2\_LIKE) .QHSSQEQTF QLLKLWKHQN KDQDIV.... .KKIIQDIDL CENSVOHRHIG  
 tnfr2.msf(TNFR2\_HUMAN) LQREAKVPHL PADKARGTQG PEQQHLLITA PSSSSSSLES SASALDRRAP  
 Consensus -Q----- --K-----Q-----R-----

351 400  
 tnfr2.msf(TNFR2\_LIKE) HANLTFEQLR SLMESLPGKK VGEADIEKTI KACKPSDQIL KLLSLWRIKN  
 tnfr2.msf(TNFR2\_HUMAN) TRNQP..QAP GVEASGAGEA RASTGSSDSS PGHGTQ..V NVTCIVNVCS  
 Consensus --N-----Q-----S-----G-----

401 450  
 tnfr2.msf(TNFR2\_LIKE) GDQDTLKGLM HALKHSKTYH FPTNCHSESK EDHQVPSQLH NVQIVSEVIF  
 tnfr2.msf(TNFR2\_HUMAN) SSDHSSQCSS QA...SSTMG DTDSSPESP KDEQVPFSKE ECAFRSOLET  
 Consensus ----- --A-----S-T-----SES--D-QVP-----S-----

451 475  
 tnfr2.msf(TNFR2\_LIKE) RNRD.....  
 tnfr2.msf(TNFR2\_HUMAN) PETLLGSTEE KPLPLGVPDA GMKPS  
 Consensus -----

MATCH WITH FIG. 2C

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# FIG. 2C

MATCH WITH FIG. 2B

Query: 38 QLLCDKCPFGTYLKQHCTAKWKTVCAPCPDHYTDSWHTSDECLYCSVPVCKELQVVKQEC 97

Q+ C KC PG + K CT TVC C D YT W+ ECL C C Q Q-C

Sbjct: 29 QMCCSKCSPGQHAKVFCTKTSITVCDSCEDSTYTQLANNWVPECLSCGSRCSDDQVETQAC 88

Query: 98 NRTHNRVCECKEGRYLEIEFCLKHRSCPP 126

R NR+C C+ G Y + R C P

Sbjct: 89 TREQNRICTRPGWYCALSKQEGCRICAP 117

Query: 118 CLKHRSCPPGFGVWQAGTPERNIVCKRCPDGFESNETSSKAPCRKHTNCSVFL 171

C R C PGFV + GT + VCK C G FSN TSS CR H C+V +

Sbjct: 115 CAPLRKCRPGFVARPGTETSDVCKPCAPGTFSNITSSDIDCRPHQICNVVAI 168

Query: 177 GNATHINICSGNS 189

GNA+ D +C+ S


Sbjct: 170 GNASMDAVCTSTS 182

Query: 363 SESKEDHQVP 372

SES +D QVP

Sbjct: 391 SESPKDEQVP 400

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BAR CODE LABEL		U.S. PATENT APPLICATION			
					
SERIAL NUMBER		FILING DATE	CLASS	GROUP ART UNIT	
08/469,637		06/06/95	435	1806	
APPLICANT	JOHN M. GREENE, GAITHERSBURG, MD; ROBERT D. FLEISCHMANN, GAITHERSBURG, MD.				
	**CONTINUING DATA***** VERIFIED  _____				
	**FOREIGN/PCT APPLICATIONS***** VERIFIED  _____				
STATE OR COUNTRY	SHEETS DRAWING	TOTAL CLAIMS	INDEPENDENT CLAIMS	FILING FEE RECEIVED	ATTORNEY DOCKET NO.
MD	5	20	3	\$730.00	325800-381
ADDRESS	GREGORY D FERRARO C/O CARELLA BYRNE BAIN GILPILLAN CECCHI STEWART AND OLSTEIN 6 BECKER FARM ROAD ROSELAND NJ 070168				
	HUMAN TUMOR NECROSIS FACTOR RECEPTOR				
TITLE					
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